81

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 982 406 A2

(12) EUROPEAN PATENT APPLICATION

(43) Date of publication:
01.03.2000 Bulletin 2000/09

(51) Int. Cl.[7]: C12P 7/26, C12N 1/20
// (C12P7/26, C12R1:15),
(C12P7/26, C12R1:06)

(21) Application number: 99115723.1

(22) Date of filing: 10.08.1999

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU
MC NL PT SE
Designated Extension States:
AL LT LV MK RO SI

(30) Priority: 19.08.1998 EP 98115564

(71) Applicant:
F. HOFFMANN-LA ROCHE AG
4070 Basel (CH)

(72) Inventors:
- Shimizu, Sakaya
Kyoto-shi, Kyoto-fu (JP)
- Wada, Masaru
Yoshida-gun, Fukui-ken (JP)

(54) **Microbial production of actinol**

(57) A process for the manufacture of (4R, 6R)-4-hydroxy-2,2,6-trimethylcyclohexanone comprises contacting (6R)-2,2,6-trimethylcyclohexanedione with a microorganism which is selected from the group consisting of microorganisms of the genera *Cellulomonas*, *Corynebacterium*, *Planococcus* and *Arthrobacter* and which is capable of the selective asymmetric reduction of (6R)-2,2,6-trimethylcyclohexanedione to (4R, 6R)-4-hydroxy-2,2,6-trimethylcyclohexanone, and recovering the resulting (4R, 6R)-4-hydroxy-2,2,6-trimethylcyclohexanone from the reaction mixture. The selective asymmetric reduction can be effected in the presence of a co-factor such as nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), or said co-factor with glucose and glucose dehydrogenase (GDH), and/or in the presence of a surfactant. The product is useful for the synthesis of carotenoids such as zeaxanthin.

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Description

[0001] The present invention relates to a process for the microbial production of (4R,6R)-4-hydroxy-2,2,6-trimethylcyclohexanone (hereinafter referred to as actinol) from (6R)-2,2,6-trimethylcyclohexanedione (hereinafter referred to as levodione). Actinol is useful for the synthesis of carotenoids, such as zeaxanthin. More particularly, the present invention relates to a process for the microbial production of actinol utilizing a specific microorganism which is capable of selectively asymmetrically reducing the carbonyl group at the C-4 position of levodione.

[0002] Actinol has previously been prepared by the optical resolution of the diastereomeric mixture of actinol. Such process, however, requires hydrogenation of levodione by metal catalysts, and subsequent optical resolution by chemical means with resolving agents such as maleic anhydride (T. Ohashi et al., the proceedings of the symposium "Molecular Chirality 1996" held on May 30 and 31, 1996, in Tokyo, Japan, pages 47 to 50, "Practical Syntheses using Biocatalysts"). Accordingly, this process is not economically feasible for industrial purposes.

[0003] Processes of enzymatic preparation of actinol from levodione per se are known. For example, *Bacillus thermophilus* is capable of converting racemic dihydrooxoisophorone to 4 isomers of 4-hydroxy-2,2,6-trimethylcyclohexanone, i.e. to the cis-(4R,6S)-, cis-(4S,6R)-, trans-(4R,6R)- and trans-(4S,6S)-isomers. The resulting quantitative ratio of these isomers is 68:25:5:2 (J. Biotechnol., 9(2), 117-128, 1989). Accordingly, the content of the (4R,6R)-isomer, actinol, is only 5% of the total isomers, and this process is also not economically feasible for industrial purposes.

[0004] As a result of extensive studies on selective asymmetric reduction of levodione, it has now been found that actinol can be obtained efficiently from levodione by selective asymmetric reduction using certain microorganisms, followed by recovery of the actinol from the reaction mixture. The present invention is based upon this finding.

[0005] Accordingly, the present invention provides a process for the manufacture of actinol which comprises contacting levodione with a microorganism which is selected from the group consisting of microorganisms of the genera *Cellulomonas*, *Corynebacterium*, *Planococcus* and *Arthrobacter* and which is capable of selective asymmetric reduction of levodione to actinol, and recovering the resulting actinol from the reaction mixture.

[0006] A screening was effected using a method known per se. For example, a microorganism is cultivated in a nutrient medium containing saccharides such as glucose and sucrose, alcohols such as ethanol and glycerol, fatty acids such as oleic acid and stearic acid or esters thereof, or oils such as rapeseed oil and soybean oil as carbon sources; ammonium sulfate, sodium nitrate, peptone, amino acids, corn steep liquor, bran, yeast extract and the like as nitrogen sources; magnesium sulfate, sodium chloride, calcium carbonate, potassium monohydrogen phosphate, potassium dihydrogen phosphate and the like as inorganic salt sources; and malt extract, meat extract and the like as other nutrient sources by a conventional method to provide cells. The cultivation can be carried out aerobically, normally for a cultivation period of 1 to 7 days at a medium pH of 3 to 9 and a cultivation temperature of 10 to 40°C. After the cultivation, the resulting cells are collected by centrifugation or filtration. The cells thus obtained and levodione are brought (contacted) together in a solvent such as water, potassium phosphate buffer, acetonitrile, ethanol and the like, and a reaction is initiated under appropriate reaction conditions (levodion concentration: 400 to 2000 mg/g dry cells/l, pH range: 4 to 9, temperature range: 20 to 50°C, reaction period: 10 minutes to 80 hours). The reaction mixture is extracted with an organic solvent such as ethyl acetate, n-hexane, toluene, n-butyl acetate and the like. The extracted solution is subjected to an appropriate chromatography to measure the productivity of actinol.

[0007] As a result of the screening, it has been found that microorganisms belonging to the genera *Cellulomonas*, *Corynebacterium*, *Planococcus* and *Arthrobacter* are capable of the selective asymmetric reduction of levodione. Especially preferred such microorganisms are *Cellulomonas* sp. AKU672, *Corynebacterium aquaticum* AKU610, *Corynebacterium aquaticum* AKU611, *Planococcus okeanokoites* AKU152 and *Arthrobacter sulfureus* AKU635, especially the first three named microorganisms. Of the five named microorganisms, *Corynebacterium aquaticum* AKU611 is the most preferred.

[0008] The microorganisms *Cellulomonas* sp. AKU672, *Corynebacterium aquaticum* AKU610 and *Corynebacterium aquaticum* AKU611 were isolated from soil samples collected at Lake Manahime, Fukui Prefecture, Japan. These micoorganisms were deposited with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan on August 4, 1998 under the Budapest Treaty and have the following designations:

Cellulomonas sp. AKU672 (FERM BP-6449)
Corynebacterium aquaticum AKU610 (FERM BP-6447)
Corynebacterium aquaticum AKU611 (FERM BP-6448)

[0009] These three microorganisms, and also *Planococcus okeanokoites* AKU152 and *Arthrobacter sulfureus* AKU635, are new and represent a further aspect of the present invention.

[0010] The above-mentioned strain AKU672 (FERM BP-6449) has the following taxonomical properties:

[0011] Typical pleomorphism of strain *Cellulomonas* sp. AKU672 was found on electron microscopic observation. An old culture of the strain was coccoidal as shown in Fig. 1. In young cultures, irregular rods were dominant (Fig. 2). The

morphological, physiological and biochemical characteristics of the strain are summarized in Tables I and II.

Table I

Morphological and Cultural Characteristics of Strain *Cellulomonas* sp. AKU672	
Form and size	Coryneform
	Old culture; coccoid cells, ca. 0.5-0.6 μm
	Fresh culture; irregular rods, 0.5-0.7 μm by 20 or more μm
Motility	Motile with one flagellum
Gram strain	+
Spores	No observation
Nutrient agar plate	Circular, convex, smooth, entire, yellow (2 days)
Nutrient broth	Ring and slight sediment
Gelatin stab	Liquefaction
Litmus milk	Acid formation
Relation to NaCl	Grow up to 5% NaCl

Table II

Physiological and Biochemical Characteristics of Strain *Cellulomonas* sp. AKU672	
Type of cell wall	Ornithine
Type of cell division	Bending
GC content (%)	74.7%
Hydrolysis of gelatin	+
Hydrolysis of starch	+
Production of indole	-
Production of hydrogen sulfide	-
Reduction of nitrate to nitrite	+
Utilization of citrate	-
Catalase activity	+
Oxidase activity	-
Urease activity	-
DNase activity	+
Amino peptidase activity	-
Cellulose attack	-
Voges-Proskauer test	-
Methyl-red test	-
Oxidation-Fermentation test *	Fermentation
Cleavage of carbohydrates	Acid production but no gas from arabinose, arbutin, cellobiose, dextrin, fructose, galactose, glucose, glycogen, maltose, starch, sucrose, trehalose and xylose;
	No acid production from glycerol, inulin, lactose, mannitol, mannose, α-methylglucoside, raffinose, rhamnose, sorbitol and sorbose
Optimum temperature for growth	37-42°C
Optimum pH for growth	pH 6.0-7.5
Heating at 63°C for 30 min. in skimmed milk	Survives
Aerobic or anaerobic	Aerobic

* R. Hugh & E. Leifson, J. Bacteriol. 66, 24(1953)

[0012] The strain *Cellulomonas* sp. AKU672 is gram-positive and aerobic, and can be classified as belonging to the group of "coryneform bacteria". This strain was motile with one flagellum. Ornithine was found in the cell wall as the principal amino acid. Its content according to gas chromatography (GC) was found to be 74.7%. Bending-like cell division was observed. The strain produced acid from a wide variety of sugars without gas formation for 4 days. This strain did not show cellulolytic activity.

[0013] The classification of coryneform bacteria is not well established. Recently, Yamada and Komagata [J. Gen. Appl. Microbiol., 18, 417(1992)] proposed classifying the coryneform bacteria into seven groups depending on the principal type of cell division, cell wall composition and DNA content according to GC. They differentiated Group 4 from other groups despite the lack of cellulolytic activity. Bacteria of this group exhibit the bending type of cell division, and the principal amino acid in the cell wall is ornithine. Their contents according to GC are distributed in a narrow and high range from 71 to 73%. These bacteria produce acid fermentatively from a wide variety of sugars. According to their proposal, the strain *Cellulomonas* sp. AKU672, which did not show cellulolytic activity, should belong to Group 4. Other characteristics of the strain on classification coincided well with those of Group 4, and so it has been tentatively named

as *Cellulomonas* sp. AKU672.

[0014] The above-mentioned strains AKU610 and AKU611 have the following taxonomical properties :

1) Growable temperature:	15-40°C
2) Optimum temperature for growth:	30°C
3) Obligatory aerobic and gram negative microorganism	
4) Spore formation:	None
5) Polymorphism and traditional rod-cocus cycles can be observed during cultivation.	
6) Motility:	None

[0015] Moreover, the strains *Corynebacterium aquaticum* AKU610 and AKU611 were identified as such based on assimilation of various carbon sources by the Biolog System (Biolog Inc., 3447 Investment Blvd., Suite 3, Hayward, California 94545, USA : Nature Vol. 339, 157-158, May 11, 1989) as follows:

[0016] Cells of each strain were inoculated with 96-well microtiter-plates and incubated for 24 hours at 28°C. Each well contains one of 96 kinds of carbon sources in BUGM+B medium (Biolog Universal Growth Media + blood; Biolog Inc.).

[0017] After incubation, each strain showed the following assimilation of carbon sources:

C source	AKU610	AKU611	C source	AKU 610	AKU611
A1	-	-	A2	-	-
A3	-	-	A4	-	-
A5	-	-	A6	-	-
A7	-	-	A8	+	+
A9	+	+	A10	-	-
A11	-	-	A12	+	+
B1	-	-	B2	-	+
B3	-	-	B4	+	-
B5	+	+	B6	-	-
B7	+	+	B8	-	-
B9	+	+	B10	+	+
B11	+	+	B12	-	-
C1	-	-	C2	-	-
C3	-	-	C4	+	+
C5	+	+	C6	+	+
C7	+	-	C8	+	+
C9	-	-	C10	-	-
C11	-	-	C12	-	-
D1	-	-	D2	-	-
D3	+	+	D4	-	-
D5	+	+	D6	-	-
D7	-	-	D8	-	+
D9	-	-	D10	-	-
D11	+	+	D12	+	+
E1	-	-	E2	-	-
E3	+	+	E4	-	-
E5	-	-	E6	-	-
E7	-	-	E8	-	-
E9	-	-	E10	-	-
E11	-	-	E12	-	-
F1	-	-	F2	-	-

F3	-	-	F4	-	-
F5	-	-	F6	+	+
F7	-	-	F8	-	-
F9	-	-	F10	-	-
F11	-	-	F12	-	-
G1	-	-	G2	-	-
G3	-	-	G4	-	-
G5	-	-	G6	-	-
G7	-	-	G8	-	-
G9	-	-	G10	-	-
G11	-	-	G12	-	-
H1	-	-	H2	-	-
H3	-	-	H4	-	-
H5	-	-	H6	-	-
H7	-	-	H8	-	-
H9	-	-	H10	-	-
H11	-	-	H12	-	-

A1 : water A2 : α-cyclodextrin
A3 : β-cyclodextrin A4 : dextrin
A5 : glycogen A6 : inulin
A7 : mannan A8 : Tween® 40
A9 : Tween® 80 A10 : N-acetyl-D-glucosamine
A11 : N-acetyl-D-mannosamine
A12 : amygdalin B1 : L-arabinose
B2 : D-arabitol B3 : arbutin
B4 : cellobiose B5 : D-fructose
B6 : L-fucose B7 : D-galactose
B8 : D-galacturonic acid B9 : gentiobiose
B10 : D-gluconic acid B11 : α-D-glucose
B12 : m-inositol C1 : α-D-lactose
C2 : lactulose C3 : maltose
C4 : maltotriitrose C5 : D-mannitol
C6 : D-mannose C7 : D-melezitose
C8 : D-melibiose C9 : α-methyl D-galactoside
C10 : α-methyl-D-galactoside C11 : 3-methyl-glucose

C12 : α-methyl-D-glucoside	D1 : β-methyl D-glucoside
D2 : α-methyl D-mannoside	D3 : palatinose
D4 : D-psicose	D5 D-raffinose
D6 : L-rhamnose	D7 : D-ribose
D8 : salicin	D9 : sedoheputulosan
D10 : D-sorbitol	D11 : stachyose
D12 : sucrose	E1 : D-tagatose
E2 : D-trehalose	E3 : turanose
E4 : xylitol	E5 : D-xylose
E6 : acetic acid	E7 : α-hydroxybutyric acid
E8 : β-hydroxybutyric acid	E9 : γ-hydroxybutyric acid
E10 : p-hydroxyphenylacetic acid	
E11 : α-keto-glutaric acid	E12 : α-keto-valeric acid
F1 : lactamide	F2 : D-lactic acid methyl ester
F3 : L-lactic acid	F4 : D-malic acid
F5 : L-malic acid	F6 : methyl pyruvate
F7 : monomethyl succinate	F8 : propionic acid
F9 : pyruvic acid	F10 : succinamic acid
F11 : succinic acid	F12 : N-acetyl-L-glutamic acid
G1 : alaninamide	G2 : D-alanine
G3 : L-alanine	G4 : L-alanyl-glycine
G5 : L-asparagine	G6 : L-glutamic acid
G7 : glycyl-L- glutamic acid	G8 : L-pyloglutamic acid
G9 : L-serine	G10 : putrscine
G11 : 2,3-butanediol	G12 : glycerol
H1 : adenosine	H2 : 2'-deoxy adenosine
H3 : inosine	H4 : thymidine
H5 : uridine	H6 : adenosine-5'-monophosphate
H7 : thymidine-5'-monophosphate	
H8 : uridine-5'-monophosphate	
H9 : fructose-6-phosphate	H10 : glucose-1-phosphate
H11 : glucose-6-phosphate	H12 : DL-α-glycerol phosphate

[0018] From the above results, both strains are identified as *Corynebacterium aquaticum* and named *Corynebacterium aquaticum* AKU610 and AKU611, respectively.

[0019] Other microorganisms mentioned above are available from a public depositary (culture collection) to anyone upon request, such as the Institute of Fermentation Osaka, Japan (IFO). Examples of such deposited strains are *Planococcus okeanokoites* AKU152 (IFO 15880) and *Arthrobacter sulfureus* AKU635 (IFO 12678).

[0020] The selective asymmetric reduction process of the present invention can be carried out batchwise, semibatchwise or continuously in water or in general in a solvent medium which is miscible with water, enhances levodione solubility and is inert to the enzyme reaction, such as 0.01 to 0.5M potassium phosphate buffer, another buffer with the pH range 4 to 10, acetonitrile, ethanol or N,N-dimethylformamide. The concentration of levodione is conveniently 400 to 2000 mg/1g dry cells/l, preferably 400 to 800 mg/1g dry cells/l. The selective asymmetric reduction process may be carried out in a pH range from 4 to 9, preferably from 6 to 7, in a temperature range from 20 to 50°C, preferably 30 to 40°C, and for 10 minutes to 80 hours, preferably for 8 hours to 24 hours.

[0021] The selective asymmetric reduction process of the present invention is conveniently carried out in the presence of a co-factor such as nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), or said co-factor with glucose and glucose dehydrogenase (GDH). The concentration of such co-factor in the reaction medium is preferably 300 mM/l or more, more preferably from 700 mM/l to 900 mM/l. Moreover, the yield of actinol can be increased by addition of a surfactant to the reaction mixture. Span® 20, Span® 80, Tween® 20, Tween® 40 (all available from Wako Pure Chemical Ind., 3-1-2 Dosho-machi, Osaka, Japan) and the like are examples of surfactants which can be used. The amount of surfactant in the reaction medium is conveniently 2 to 20 mM/l, preferably about 8 mM/l.

[0022] After selective asymmetric reduction has been completed, the actinol thus obtained can be recovered by extraction with a water-insoluble (water-immiscible) organic solvent which readily solubilizes actinol, such as ethyl acetate, n-hexane, toluene or n-butyl acetate. Further purification of actinol can be effected by concentrating the extract to directly crystallize the actinol or by the combination of various kinds of chromatography, such as thin layer chromatography, adsorption chromatography, ion-exchange chromatography and/or gel filtration chromatography. If necessary, high performance liquid chromatography can also be applied. A preferred recovery leading to crystals of actinol involves extracting the actinol with ethyl acetate and concentrating the extract to obtain crystals of actinol.

[0023] As an alternative to the above described "resting cell reaction" technique, actinol can be produced by fermentation of the above microorganisms in a nutrient medium in the presence of levodione, i.e. in a "growing cell reaction". Both alternatives are embraced by the process of the present invention.

[0024] As nutrient media in the "growing cell reaction" technique there may be used those which contain saccharides such as glucose and sucrose, alcohols such as ethanol and glycerol, fatty acids such as oleic acid and stearic acid or esters thereof, or oils such as rapeseed oil and soybean oil as carbon sources; ammonium sulfate, sodium nitrate, peptone, amino acids, corn steep liquor, bran, yeast extract and the like as nitrogen sources; magnesium sulfate, sodium chloride, calcium carbonate, potassium monohydrogen phosphate, potassium dihydrogen phosphate and the like as inorganic salts; and malt extract, meat extract and the like as other nutrient sources. As a further aspect of the present invention, actinol can be produced by fermentation of the above microorganisms in a nutrient medium in the presence of levodione.

[0025] The fermentation can be carried out aerobically, normally for an incubation period of 1 to 7 days at a medium pH of 3 to 9 and a fermentation temperature of 10 to 40°C.

[0026] The microorganisms to be used in the fermentation may be in any form, for example, cultures obtained by fermentation of strains in liquid media, cells separated from liquid cultures, dried cells obtained by processing cells or cultures, or immobilized cells.

[0027] The following Examples illustrate the present invention.

Example 1

[0028] A liquid medium (pH 7.0) consisting of 0.5% 1,4-cyclohexanedione (structurally analogous to (6R)-2,2,6-trimethylcyclohexanedione; used for the screening), 0.5% Tween® 20, 0.1% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$ and 0.02% yeast extract was dispersed in 5 ml portions into test tubes, and then sterilized at 121°C for 20 minutes. About 0.3 g of soil sample was introduced into each of these tubes and cultivated for 24 hours at 30°C. A 0.1 ml portion of the culture thus obtained was used to inoculate fresh test tube medium as above, and this operation was repeated twice to enrich objective microorganisms. The enriched culture thus obtained was diluted with saline and spread on an agar medium consisting of the same ingredients as above. Simultaneously, supernatant of the soil suspension in saline was appropriately diluted and spread on the agar medium as well. The plates were incubated for 48 hours at 30°C. Grown colonies on the plates were used to inoculate 5 ml liquid medium (pH 7.0) consisting of 1.0% glucose, 0.3% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 1.5% peptone (Mikuni Kagaku Sangyo K.K., 4-1-6 Muro-machi, Nihonbashi, Chuo-ku,Tokyo, Japan), 0.2% NaCl and 0.1% yeast extract (Nacalai Tesuque Inc., Karasumaru Nishihairu, Nijohtouri, Nakakyo-ku, Kyoto, Japan) in a tube. After the tubes had been incubated at 30°C for 24 hours, cells were collected by centrifugation and washed with saline. The cells thus obtained were subjected for the subsequent screening. In addition to the above microorganisms, air-dried cells of the microorganisms that had been cultivated in a nutrient medium were also used for the screening.

Example 2

[0029] A reaction mixture (pH 7.0 in 0.1 M potassium phosphate buffer) containing 0.6 mg of NAD (Oriental Yeast Co., 3-6-10 Azusawa, Itabashi-ku, Tokyo, Japan), 0.6 mg of NADP (Oriental Yeast Co.), 50 mg of D-glucose and 0.2 mg of D-glucose dehydrogenase (Amano Pharmaceutical Co., 1-2-7 Nishiki, Naka-ku, Nagoya, Japan) was prepared. About 0.3 g of the cells prepared in Example 1 was added to 1 ml of the reaction mixture, followed by a sufficient amount of (6R)-2,2,6-trimethylcyclohexanedione to give a final concentration of 0.5%. The reaction mixture was then incubated with shaking for 24 hours at 30°C. After the incubation, the reaction mixture was extracted with 1 ml of ethyl acetate and concentrated. The yield and the optical purity of the (4R, 6R)-4-hydroxy-2,2,6-trimethyl-cyclohexanone were analyzed by gas chromatography [column: HR-20M (Shinwa Chemical Ind., Keishyo-cho 50, Fushimi-ku, Kyoto, Japan) 0.25 mmϕ x 30m, column temperature: 160°C (constant), injector temperature: 250°C, carrier gas: He (approx. 1ml/min)]. The results are presented in Table III.

Table III

Strain Name	Rate of reduction (%)	Optical purity of (4R,6R)-4-hydroxy-2,2,6-trimethyl-cyclohexanone (% e.e.)
Planococcus okeanokoites AKU152 (IFO 15880)	42.3	56.7
Arthrobacter sulfureus AKU635 (IFO 12678)	64	44
Cellulomonas sp. AKU672 (FERM BP-6449)	73	78.3
Corynebacterium aquaticum AKU610 (FERM BP-6447)	93.7	85.9
Corynebacterium aquaticum AKU611 (FERM BP-6448)	97.4	87.7

Example 3

[0030] The effect of the addition of NAD or NADP to the reaction mixture was elucidated by using the microorganisms given in Table III. The basic reaction mixture contained all the components described in Example 2 except NAD and NADP. The cells of the microorganisms used in the present Example were air-dried, and 10 mg of the cell mass were incorporated into the reaction mixture. The reaction was carried out at 30°C for 24 hours. The results are presented in Table IV, in which the optical purity (% e.e.) values apply to the (4R,6R)-isomer, as is also the case in Tables V (Example 4) and VI (Example 5).

Table IV

	Co-factor Addition					
	NAD		NADP		None	
Strain Name	Rate of reduction (%)	Optical purity(% e.e.)	Rate of reduction (%)	Optical purity(% e.e.)	Rate of reduction (%)	Optical purity(% e.e.)
Planococcus okeanokoites AKU152 (IFO 15880)	89.3	60.4	65.4	54.7	63.4	58.2
Arthrobacter sulfureus AKU635 (IFO 12678)	82.7	24	66.5	-7.3	56.5	-9.5
Cellulomonas sp. AKU672 (FERM BP-6449)	59.2	67.1	30.1	21.6	24.8	25.7

Table IV (continued)

	Co-factor Addition					
	NAD		NADP		None	
Strain Name	Rate of reduction (%)	Optical purity(% e.e.)	Rate of reduction (%)	Optical purity(% e.e.)	Rate of reduction (%)	Optical purity(% e.e.)
Corynebacterium aquaticum AKU610 (FERM BP-6447)	62.5	87.4	60	85.3	17	52.1
Corynebacterium aquaticum AKU611 (FERM BP-6448)	96.8	93.9	85.3	88.5	92.5	89.1

Example 4

[0031] The effect of the addition of various surfactants (final concentration: 0.1 w/v %) in the reaction mixture was elucidated by using the microorganisms given in Table III. The basic reaction mixture contained all the components described in Example 2. The cells of the microorganisms used in the present Example were air-dried, and 10 mg of the cell mass were incorporated into the reaction mixture. The reaction was carried out at 30°C for 24 hours. The results are presented in Table V.

Table V

	Surfactant					
	None		Tween® 20		Tween® 40	
Strain Name	Rate of reduction (%)	Optical purity(% e.e.)	Rate of reduction (%)	Optical purity(% e.e.)	Rate of reduction (%)	Optical purity(% e.e.)
Planococcus okeanokoites AKU152 (IFO 15880)	53.9	51.6	78.1	63.4	63.9	57.7
Arthrobacter sulfureus AKU635 (IFO 12678)	73.4	25.1	86.8	38.6	82.4	33.8
Cellulomonas sp. AKU672 (FERM BP-6449)	32.3	80	32.1	86.2	not measured (n.m.)	n.m.
Corynebacterium aquaticum AKU610 (FERM BP-6447)	58.9	87.6	71.7	89.3	n.m.	n.m.
Corynebacterium aquaticum AKU611 (FERM BP-6448)	85.7	92.6	97.5	93.7	n.m.	n.m.

Table V (continued)

	Surfactant			
	Span® 20		Span® 80	
Strain Name	Rate of reduction (%)	Optical purity (% e.e.)	Rate of reduction (%)	Optical purity (% e.e.)
Planococcus okeanokoites AKU152 (IFO 15880)	71.4	65.7	57.3	57.3
Arthrobacter sulfureus AKU635 (IFO 12678)	78.5	49.9	65.2	32
Cellulomonas sp. AKU672 (FERM BP-6449)	22.2	66.2	38	78.1
Corynebacterium aquaticum AKU610 (FERM BP-6447)	64.6	89.9	83	87.3
Corynebacterium aquaticum AKU611 (FERM BP-6448)	96.7	94	88.8	93.2

Example 5

[0032] The influence of the substrate concentration on the reaction was elucidated at concentrations of 0.5, 1.0 and 1.5%. The basic reaction mixture contained all the components described in Example 2. In the present Example, the

cells of *Corynebacterium aquaticum* AKU611 (FERM BP-6448) were air-dried, and 10 mg of the cell mass were incorporated into the reaction mixture. The reaction was carried out at 30°C for 24 hours. The results are represented in Table VI.

Table VI

Substrate Concentration (%)	Rate of reduction (%)	Optical purity (% e.e.)	Product concentration (%)
0.5	92.2	93.0	0.46
1.0	73.1	92.9	0.73
1.5	66.3	92.8	0.99

Example 6

[0033] *Corynebacterium aquaticum* AKU611 (FERM BP-6448) was cultivated for 24 hours at 30°C in 20 l of the culture medium composed of 0.1% yeast extract, 1.5% peptone, 2.0% D-glucose, 0.02% $MgSO_4 \cdot 7H_2O$, 0.3% K_2HPO_4 and 0.2% NaCl using a 30 l jar fermentor with agitation at 400 rpm and aeration of 0.5 l per minute. Cells were collected from the culture by centrifugation at 5,000g for 5 minutes thereafter. The weight of the paste of cells thus obtained was 400 g.

[0034] Then, 12 g of levodione and 120 g of D-glucose were added to the cell paste and the volume was brought to 2.4 l with ion exchanged water. The pH was adjusted to 7.0 with 2.0% NaOH solution. The reaction mixture was transferred into a 2 l flask and incubated at 30°C for 15 hours with shaking at 220 rpm. After the incubation, the reaction mixture was separated by centrifugation at 12,000g for 5 minutes. The volume of the reaction mixture thus obtained was 2.2 l, and the optical purity, the yield and the concentration of actinol were 96% e.e., 93% and 4.6 g/l, respectively.

Example 7

[0035] The reaction mixture (10 l), prepared as described in Example 6, was mixed with ethyl acetate (10 l) to extract actinol. The ethyl acetate phase (7.5 l) was separated and 350 g of active carbon powder were added thereto for decolorizing it. After 10 minute stirring, the carbon powder was removed by filtration. 600 g of anhydrous Na_2SO_4 were added to the 6.5 l of ethyl acetate solution for dehydration. After a few minutes stirring, Na_2SO_4 was removed by filtration. The ethyl acetate solution (6.0 l) was concentrated to 50 ml under reduced pressure at 30°C. To the concentrate thus obtained there were added 5 l of n-hexane, and the mixture was stirred for five minutes, then cooled to 5°C and maintained at this temperature for 12 hours to crystallize actinol. The crystallized actinol was collected by filtration and then dried. The weight of actinol crystals thus obtained was 32 g, and the purity, the optical purity and the yield of actinol were 96%, 96% e.e and 70%, respectively.

Example 8

[0036] Seed culture broth (150ml) of *Corynebacterium aquaticum* AKU611 (FERM BP-6448) was inoculated into 3 l of the fermentation medium composed of 0.1% yeast extract, 1.5% peptone, 2.0% glucose, 0.02% $MgSO_4 \cdot 7H_2O$, 0.3% K_2HPO_4, 0.2% NaCl and 0.3% levodione. The fermentation was carried out for 48 hours at 30 using a 5 l jar fermentor with agitation at 250 rpm and aeration of 1.5 l per minute. The pH of the fermentation broth was controlled at 7.0 by NH_3 gas. After the fermentation, the broth was removed and the cells were collected by centrifugation at 12,000g for 5 minutes. The optical purity, the yield and the concentration of actinol in the broth were 96% e.e., 71% and 2.1 g/l respectively.

Claims

1. A process for the manufacture of (4R, 6R)-4-hydroxy-2,2,6-trimethylcyclohexanone which comprises contacting (6R)-2,2,6-trimethylcyclohexanedione with a microorganism which is selected from the group consisting of microorganisms of the genera *Cellulomonas*, *Corynebacterium*, *Planococcus* and *Arthrobacter* and which is capable of the selective asymmetric reduction of (6R)-2,2,6-trimethylcyclohexanedione to (4R, 6R)-4-hydroxy-2,2,6-trimethylcyclohexanone, and recovering the resulting (4R, 6R)-4-hydroxy-2,2,6-trimethylcyclohexanone from the reaction mixture.

2. A process according to claim 1, wherein the microorganism is selected from the group consisting of *Cellulomonas* sp. AKU672 (FERM BP-6449), *Corynebacterium aquaticum* AKU610 (FERM BP-6447), *Corynebacterium aquaticum* AKU611 (FERM BP-6448) and is preferably *Corynebacterium aquaticum* AKU611 (FERM BP-6448).

3. A process according to claim 1, wherein the microorganism is *Planococcus okeanokoites* AKU152 (IFO 15880) or *Arthrobacter sulfureus* AKU635 (IFO 12678).

4. A process according to any one of claims 1 to 3, wherein the selective asymmetric reduction is effected in the presence of NAD, NADP, or NAD or NADP with glucose and GDH.

5. A process according to any one of claims 1 to 4, wherein the selective asymmetric reduction is effected in the presence of a surfactant.

6. A process according to any one of claims 1 to 5, wherein the selective asymmetric reduction is effected in a pH range from 4 to 9 , in a temperature range from 20 to 50°C , and for 10 minutes to 80 hours.

7. A process according to any one of claims 1 to 6, wherein recovering the resulting (4R, 6R)-4-hydroxy-2,2,6-trimethylcyclohexanone from the reaction mixture is effected by extracting the (4R, 6R)-4-hydroxy-2,2,6-trimethylcyclohexanone with ethyl acetate and concentrating the resulting extract to obtain crystals of (4R, 6R)-4-hydroxy-2,2,6-trimethylcyclohexanone.

8. *Cellulomonas* sp. AKU672 (FERM BP-6449), *Corynebacterium aquaticum* AKU610 (FERM BP-6447) and *Corynebacterium aquaticum* AKU611 (FERM BP-6448).

9. *Planococcus okeanokoites* AKU152 (IFO 15880) and *Arthrobacter sulfureus* AKU635 (IFO 12678).

Fig. 1. Electron-micrograph of strain Cellulomonas sp. AKU672. Strain Cellulomonas sp. AKU672 was grown on a nutrient ager slant for 4 days at 28°C. Washed cells were dried in vacuo and shadowed with chromium.

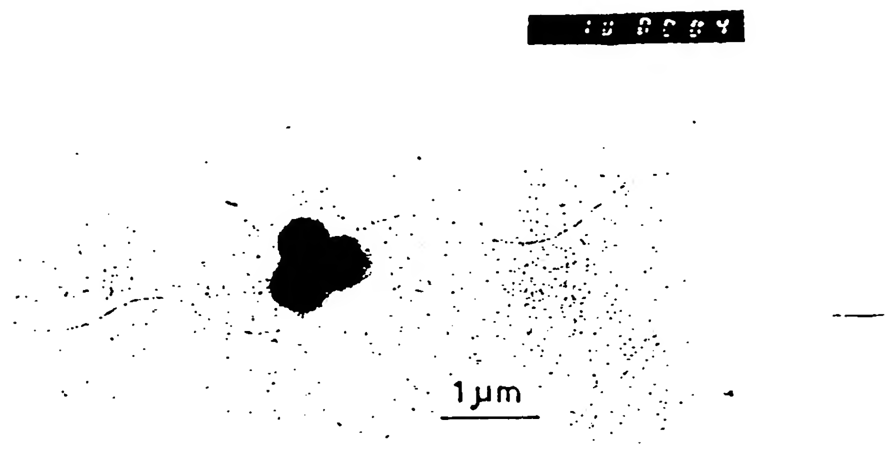
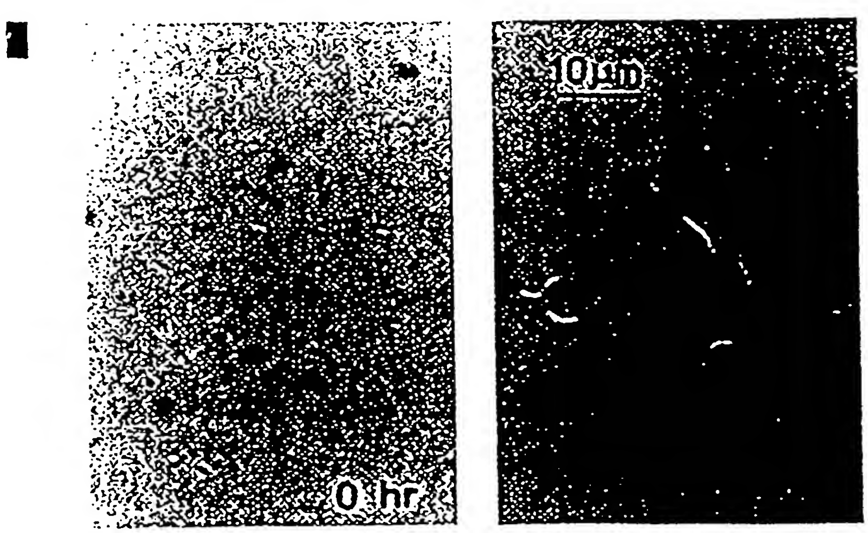


Fig. 2. Pleomorphism of strain Cellulomonas sp. AKU672. Cells were cultivated on a nutrient broth.



(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 982 406 A3

(12) EUROPEAN PATENT APPLICATION

(88) Date of publication A3:
20.09.2000 Bulletin 2000/38

(43) Date of publication A2:
01.03.2000 Bulletin 2000/09

(21) Application number: 99115723.1

(22) Date of filing: 10.08.1999

(51) Int. Cl.[7]: C12P 7/26, C12N 1/20
// (C12P7/26, C12R1:15),
(C12P7/26, C12R1:06)

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE
Designated Extension States:
AL LT LV MK RO SI

(30) Priority: 19.08.1998 EP 98115564

(71) Applicant:
F. HOFFMANN-LA ROCHE AG
4070 Basel (CH)

(72) Inventors:
- Shimizu, Sakaya
Kyoto-shi, Kyoto-fu (JP)
- Wada, Masaru
Yoshida-gun, Fukui-ken (JP)

(54) Microbial production of actinol

(57) A process for the manufacture of (4R, 6R)-4-hydroxy-2,2,6-trimethylcyclohexanone comprises contacting (6R)-2,2,6-trimethylcyclohexanedione with a microorganism which is selected from the group consisting of microorganisms of the genera *Cellulomonas*, *Corynebacterium*, *Planococcus* and *Arthrobacter* and which is capable of the selective asymmetric reduction of (6R)-2,2,6-trimethylcyclohexanedione to (4R, 6R)-4-hydroxy-2,2,6-trimethylcyclohexanone, and recovering the resulting (4R, 6R)-4-hydroxy-2,2,6-trimethylcyclohexanone from the reaction mixture. The selective asymmetric reduction can be effected in the presence of a co-factor such as nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), or said co-factor with glucose and glucose dehydrogenase (GDH), and/or in the presence of a surfactant. The product is useful for the synthesis of carotenoids such as zeaxanthin.

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2.16.7 (HRS)/3.6

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 99 11 5723

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
A,D	KUNIO NISHII ET AL: "MICROBIAL CONVERSION OF DIHYDROOXOISOPHORONE (DOIP) TO 4-HYDROXY-2,2,6-TRIMETHYLCYCLOHEXANONE (4-HTMCH) BY THERMOPHILIC BACTERIA" JOURNAL OF BIOTECHNOLOGY,NL,ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, vol. 9, no. 2, 1989, pages 117-128, XP000008425 ISSN: 0168-1656 * the whole document *	1-9	C12P7/26 C12N1/20 //(C12P7/26, C12R1:15), (C12P7/26, C12R1:06)
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TECHNICAL FIELDS SEARCHED (Int.Cl.7)
C12P
C12N

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
MUNICH	26 July 2000	Douschan, K

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26-07-2000

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